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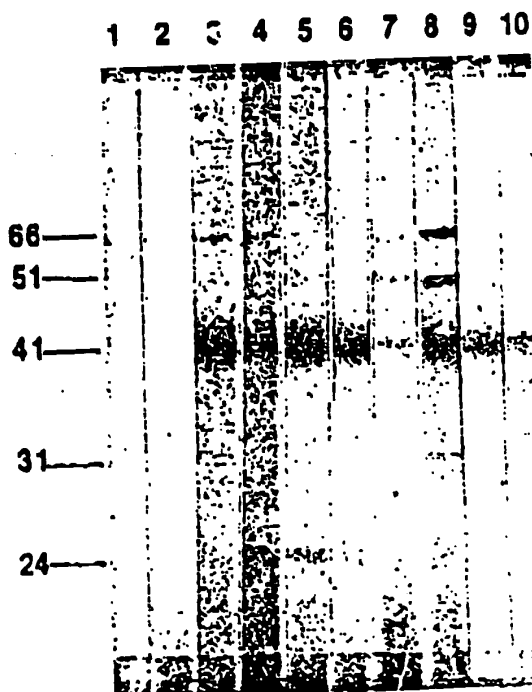
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(54) Title: ISOLATION OF PROTEINS OF HTLV-III, SEROLOGICAL DETECTION OF ANTIBODIES TO HTLV-III IN SERA OF PATIENTS WITH AIDS AND PRE-AIDS CONDITIONS, AND DETECTION OF HTLV-III INFECTION BY IMMUNO-ASSAYS USING HTLV-III AND ITS PROTEINS

(57) Abstract

Production of core antigens of the virus HTLV-III and their use in detection of antibodies in sera of AIDS and pre-AIDS patients, and to biochemical and immunological analysis of antigens associated with the virus HTLV-III. The utilization of p24 and p41 core antigens enables a greater specificity of detection. It is shown that antigens associated with the infection of human cells by the virus are specifically recognized by antibodies from AIDS patients. Specifically, HTLV-III isolated from AIDS patients and transformed by occultation with an HT cell line is specifically detected by antibodies from human sera taken from AIDS patients. The p24 and p41 antigens are used to monitor the presence of HTLV-III in cultured cells and cell-free culture fluids, where virus infection is suspected, and also to monitor antibodies in human sera. The method of detection of antibodies preferred is a radioimmunoassay (RIA), ELISA (an enzyme-linked immunosorbent assay) or indirect immunofluorescence assay.



ISOLATION OF PROTEINS OF HTLV-III,  
SEROLOGICAL DETECTION OF ANTIBODIES TO  
HTLV-III IN SERA OF PATIENTS WITH AIDS  
AND PRE-AIDS CONDITIONS, AND DETECTION  
OF HTLV-III INFECTION BY IMMUNO-ASSAYS  
USING HTLV-III AND ITS PROTEINS

Technical Field

This invention relates to the detection of antibodies in sera of AIDS and pre-AIDS patients and to biochemical and immunological analysis of HTLV-III virus and its antigens.

Background of the Invention

A family of T-lymphotropic retroviruses causes T-cell proliferation leukemia, T-cell depletion, and immunosuppression in humans infected by the viruses. These retroviruses are known as the HTLV family of T4 tropic retroviruses. Subgroup HTLV-I causes T-cell proliferation and leukemia; subgroup HTLV-II induces T-cell proliferation in vitro but its role in disease is unclear. A third group of related virus, collectively designated HTLV-III, has now been isolated from cultured cells of patients with acquired immune deficiency syndrome (AIDS). The biological properties of HTLV-III and immunological analysis of its proteins show that this virus is a member of the HTLV family. Sera of nearly 100% of patients with AIDS and of nearly 90% of homosexual men with pre-AIDS, but less than 1% of heterosexual donors, have antibodies reactive against antigens of HTLV-III. The major immune reactivity appears to be directed against p24, a 24,000 molecular weight protein believed to be a core antigen, and against p41, a 41,000 molecular weight protein believed to be an envelope antigen of the virus.

Acquired immune deficiency syndrome (AIDS) is a relatively recently recognized disease evident in several parts of the world. Its overwhelming prevalence among

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5 homosexual men with multiple sexual partners, illegal  
intravenous drug abusers, hemophiliacs, blood transfusion  
recipients, and those with close heterosexual contacts  
with members of the above high-risk groups strongly sug-  
gests that the disease spreads by the transmission of an  
infectious agent. The primary targets of affliction in  
the human body are specific subpopulations of T-cells.  
The severe immune deficiency of these patients results  
from an unusually low proportion of helper T-cells (T4)  
10 in their lymphocyte population, thus reducing the avail-  
ability of many T4 helper functions, among which is the  
production of antibodies by B-cells.

Retrovirus infection is known to lead to  
depressed immune functions in animal systems. Analogiz-  
15 ing the human response to these non-human systems, a  
human retrovirus with a tropism for T-cells was consid-  
ered a candidate in the etiology of human AIDS. As men-  
tioned above, several members of a family of human T-  
lymphotropic retroviruses (HTLV) have been isolated. One  
20 of these isolates was obtained from a black American with  
an aggressive form of T-cell lymphoma. This virus,  
designated HTLV-I, has been etiologically linked to the  
pathogenesis of adult T-cell leukemia/lymphoma (ATLL).  
In vitro infection with HTLV-I can alter T-cell function  
25 and, in some cases, leads to T-cell death. Another mem-  
ber of the HTLV family was isolated from a patient with a  
T-cell variant of hairy cell leukemia and was designated  
HTLV-II. Isolation of HTLV-I and HTLV-II have been  
reported from cultured T-cells of patients with AIDS.  
30 Isolation of another retrovirus was reported from a homo-  
sexual patient with chronic generalized lymphadenopathy,  
a syndrome that often precedes AIDS and is therefore  
referred to as "pre-AIDS." Proviral DNA of HTLV-I was  
detected in the cellular DNA of two AIDS patients, and  
35 sera of some patients were shown to react with antigens  
of HTLV-I. The correlation between AIDS and serum anti-  
bodies to HTLV-I protein is weak. The present invention

shows that the primary cause of the syndrome is a human T-lymphotropic retrovirus variant with limited cross reactivities with the known HTLV-subgroups. These new variants are designated HTLV-III. Disclosed is the use  
5 of this virus in an immunological screening of sera of patients with AIDS, pre-AIDS, and individuals at increased risk for AIDS.

HTLV-III was purified from supernatants of cell cultures supporting the continuous production of these  
10 cytopathic viruses. These HTLV variants (HTLV-III) lack immortalizing (transforming) properties for normal T-cells and mainly exhibit cytopathic effects on the T-cell helper. The cytopathic effect was overcome by finding a highly susceptible, permissive cell for cytopathic variants of HTLV, thus preserving the capacity for permanent  
15 growth after infection with the virus. These cell cultures allow for continuous production of the HTLV-III virus.

#### Disclosure of Invention

20 It is shown that antigens associated with the infection of human cells by HTLV-III virus are specifically recognized by antibodies from AIDS patients. In particular, HTLV-III isolated from AIDS patients and transmitted by cocultivation with an HT cell line, or its  
25 antigens, specifically reacts with antibodies from human sera taken from AIDS patients. Detection of the presence of the antibodies or the antigens, as desired, may be achieved by means of this reaction. This assay is very useful in monitoring HTLV-III virus infected cells and  
30 generally the cultures that contain this virus to make sure that they really produce the virus, as well as to detect AIDS antibodies in patients' sera. If there is an unidentified culture, the antigens provide one way to tell if that culture has the virus HTLV-III. A competi-  
35 tion radioimmunoassay for the antigens may be performed using the purified antigens of HTLV-III. If the culture material competes in the assay, then it means that the

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culture has this protein. If it has the protein, then it has the virus because without the virus it cannot have the protein. The detection methods preferred include radioimmunoassay (RIA), ELISA (an enzyme-linked immunosorbent assay) and ELIA (an indirect immunofluorescent assay), as well as Western Blot and Western Dot techniques. The p24 and p41 antigens are particularly preferred. The p24 antigen is a major core protein of the virus, while the p41 antigen is a major envelope antigen of the virus.

#### Brief Description of Figures

Figure 1 shows the identification of HTLV-III antigens by sera of patients in accordance with the procedure of Example 2.

Figure 2 shows protein bands caused by reaction with rabbit hyperimmune sera to HTLV-I, HTLV-II, and HTLV-III.

Figure 3 shows the SDS-polyacrylamide gel profile of a sample labeled with  $^{125}\text{I}$ .

Figure 4 shows the results of immunoprecipitation of  $^{125}\text{I}$  labeled p24 of HTLV-III with sera of patients with AIDS and AIDS-related conditions.

Figure 5A shows total competition of the precipitation of labeled p24 by unlabeled HTLV-III extract.

Figure 5B shows two cell clones infected with HTLV-III (H4/HTLV-III and H9/HTLV-III) which blocked the immunoprecipitation of HTLV-III p24 and shows that corresponding uninfected cells were totally without effect.

#### Statement of Deposit

A cell line related to the present invention, and denoted H9/HTLV-III<sub>B</sub>, has been deposited in the American Type Culture Collection (ATCC, 12301 Parklawn Drive, Rockville, Maryland 20852-1776, USA) under ATCC No. CRL 8543 on April 19, 1984. H9 is a representative and preferred cloned cell line in accordance with the invention. An additional deposit of Molt 3/HTLV-III<sub>B</sub> was made in the ATCC on August 15, 1984, under ATCC No. CRL

8602. These deposits assure permanence of the deposits and ready accessibility thereto by the public.

# Modes for Carrying Out the Invention

5 Lysates of immortalized human T-cell clones such as H9 to which HTLV-III has been transmitted by cocultivation with lymphocytes from AIDS (designated H9/HTLV-III<sub>B</sub>) were tested with human sera in a strip radioimmunoassay (RIA) based on the Western Blot technique. The sera used for the analysis were also tested  
10 by ELISA with purified HTLV-III. Sera from patients with AIDS and from some homosexuals and heroin-addicts recognized a number of specific antigens not detected by any other means. In short, the antigens associated with HTLV-III virus produced by HT cells permit the detection  
15 of antibodies in the sera of AIDS and pre-AIDS patients. The HTLV-III virus and its antigens also make possible the detection of AIDS and pre-AIDS in other samples of human sera, such as donated blood.

Also, a DNA fusion has led to the conclusion  
20 that there are at least two definite fractions to the HTLV-III, namely HTLV-III<sub>A</sub> and HTLV-III<sub>B</sub>, particularly susceptible to use in the present assay test kit procedure. Additionally, recombinant fractions of HTLV-III have been prepared. These close derivatives of HTLV-III  
25 are included within the scope of the present invention.

Prominent immune reactivity or specificity is directed against p41, a 41,000 molecular weight protein which is an envelope antigen of the HTLV-III virus, and against p24, a 24,000 molecular weight protein which is a  
30 core antigen of HTLV-III virus. The p24 antigen is called the p30 homolog because in most viruses the molecular weight is about 30,000. In bovine leukemia virus and in the HTLV series the molecular weight of the protein is about 24,000. (In testing proteins to show  
35 the relationship by amino acid sequences, the concept of homology is important. After determining the amino acid sequences of two proteins, when these sequences are lined

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up, if the same amino acids appear at the same positions in both proteins, the two proteins are said to have homology.) Sera from patients with AIDS, some homosexuals, and heroin addicts also recognize a number of other specific antigens not detected in normal sera. These antigens are about molecular weight 65,000 (p65), 60,000 (p60), and 55,000 (p55). Although other antigens were detected, these were the most significant. Example 4 provides an illustration of the specificity of these reactions.

The HTLV-III virus may be purified from an impure source of the virus, such as the tissue culture fluid, as follows. The virus is concentrated and purified with sucrose density-banding, a procedure described in J. of Virology, 38:906-915, June 1981. The viruses have their characteristic density -- type C retroviruses, of which HTLV-III is a member, have a density of 1.16 grams per cc in a sucrose solution. (The virus fraction may be located by assaying aliquots of each fraction for HTLV-III specific reverse transcriptase activity.) The cells are removed from the culture medium, giving a clarified medium. The medium is centrifuged through a continuous flow centrifuge and as the medium is loaded, because of the rotary spinning, the particles inside the sample move towards the periphery of the rotor through a sucrose gradient between 20-60% with a density in the range from 1.08 to 1.29 g/cc. These particles move towards the periphery until they reach the point in the sucrose gradient where they find their own density in the surroundings (about 36%-38% sucrose, which has the same density as about 1.16). They cannot go any farther. When all clarified culture medium has gone through the rotor, all the particles will have collected to that point (band). The centrifugation is continued so the virus reaches an equilibrium, the particles go no farther, and the fractions are collected by pumping out the contents of the rotor and collecting fractions of differ-



ent densities. Thus, the virus is purified. There are no cells in the 1.16 density fraction because the cells are removed initially, and those that may have escaped the initial removal step go farther down in the sucrose gradient since they are denser than the virus particles. There is little contamination from free proteins from the culture medium because they are lighter. The banded material in the density range of 1.15-1.18 g/cc is collected and used in the experiment.

To obtain the core protein p24, non-ionic detergents such as NP40 or Triton X100 or Tween 20, and salt (sodium chloride) are added. The combination is important because the detergent breaks up the envelope, which also contains lipids. Detergents emulsify the lipid and remove it from the structure. The virus particles are thus accessible to the sodium chloride. The ionic surroundings resulting from the addition of sodium chloride will destroy the interaction of proteins, including the core proteins, with the nucleic acid in the core. A suspension of nucleic acids and free proteins is thus obtained when the virus is treated with the detergent and salt. The ionic strength of the medium is not lowered until the nucleic acids are removed because they could recombine to form a complex. Therefore, the salt is reduced slightly to 0.3M and then passed through a column of an ion exchange resin, diethylaminoethyl cellulose (DEAE cellulose), which is a cationic matrix. It is positively charged so it will bind a negative charge. The virus extract is passed through the column of DEAE cellulose at about 0.3 M salt, which is enough to keep the nucleic acids and proteins apart. When it goes through the column, DEAE-cellulose picks up the nucleic acids, leaving the proteins to exit the column unbound.

After the nucleic acids are removed by the DEAE cellulose, the protein solution is dialyzed against a buffer. The pH is adjusted to about 6.5 and then chromatography is performed on phosphocellulose. The protein

binds to the column and is eluted with a gradient of salt. Different proteins elute at different salt concentrations. The p24 elutes between 0.25 and 0.35 molar sodium chloride. Alternately, purification may be achieved by high performance liquid chromatography (HPLC).

In summary, the process for purifying and recovering p24 viral antigen of HTLV comprises the following steps:

(1) Removing the cells from tissue culture medium;

(2) Concentrating and purifying the impure virus by sucrose density-banding;

(3) Adding a non-ionic detergent, such as Triton X100, and sodium chloride to free the nucleic acids and proteins inside the virus; and

(4) Chromatographing on DEAE cellulose (positive) and then subsequently chromatographing on phosphocellulose (cellulose phosphate resin).

Radioimmunoassay techniques for detecting antibodies include radiolabeled assays of the so-called blot technique, such as the Western Blot technique exemplified by Example 2, below.

Also operable and preferred for the detection of antibodies are the enzyme-linked immunosorbent assays (ELISA) (see Example 1). The principle of the ELISA test is the reaction of specific antibodies in sera of AIDS patients with antigens of HTLV-III (preferably previously attached to the well surface of a multiwell dish), and a subsequent reaction of the immune complex thus formed (preferably on the dish surface) with a secondary antibody reactive with human immunoglobulins. The secondary antibody is tagged with a suitable enzyme which yields a colored soluble product when incubated with an appropriate substrate. The intensity of the color yielded is a measure of the antibody titer in the patient sera and is measured using an ELISA PLATE READER.

The components of the ELISA test kit are:

1) Microtiter plates coated with HTLV-III lysate and treated with protein solutions to prevent further adsorption of proteins to the plates by means other than antigen-antibody interaction;

2) Control positive human serum of known reactivity and antibody titer to HTLV-III antigens;

3) Negative control serum with no antibody titer to HTLV-III antigens;

4) Secondary antibody reactive against human immunoglobulins, labeled with an enzyme (e.g., peroxidase, phosphatase, etc.), and a diluent solution to dilute the antibody, comprising normal serum of the animal species in which the secondary antibody is raised;

5) A substrate mixture (e.g., 0.05% orthophenylenediamine and 0.005%  $H_2O_2$ ) for peroxidase;

6) A solution to stop the enzyme reaction (e.g., 4N  $H_2SO_4$  for the peroxidase reaction with orthophenylene diamine and  $H_2O_2$ );

7) Washing buffer containing PBS and 0.02% Tween-20 (or another); and

8) Phosphate buffered saline (PBS).

The test is carried out by adding the test serum to the antigen-coated wells of the microtiter plates and incubating at room temperature for 2 hours (or longer). The wells are washed with the wash buffer and incubated with the secondary antibody with the enzyme tag for 1 hour at 37°C. The plates are washed again with wash solution and subsequently with PBS alone. The plates are then treated with the substrate solution and incubated for 10-30 minutes and the reaction stopped by adding the appropriate solution. The color yield in each well is measured by an ELISA PLATE READER.

The ELISA system above can be modified to substitute purified p24 of HTLV-III instead of lysate of HTLV-III.

Antibodies to HTLV-III may also be detected by

an indirect immunofluorescence assay. See Example 3 for an example of this technique. This assay is significant because it uses the infected T-cell as a starting material. ELISA and Western Blot techniques start with the HTLV-III virus.

As indicated above, antibodies to HTLV-III may also be detected in sera of patients with AIDS or pre-AIDS by means of the Western Blot technique (see for instance Example 2). HTLV-III is lysed and electrophoretically fractionated on a poly-acrylamide slab gel. Protein bands on the gel are then electrophoretically transferred to a nitrocellulose sheet, for instance by transverse electrophoresis. The remaining protein binding sites on the nitrocellulose sheet are saturated by incubating the sheet in a solution containing an unrelated protein such as bovine albumin, the sheet is separated into narrow strips such that each strip contains a representative profile of the viral antigens as discrete bands, and the individual strips are used as matrix-bound antigens for detecting antibodies reactive with them in serum samples. Strip solid phase radioimmunoassays have been performed. Test sera obtained from human patients suspected of having contracted AIDS are added to tubes containing the above described strips. Another antibody of  $^{125}\text{I}$  labeled goat anti-human immunoglobulin is added to the reaction strips which are then exposed to X-ray film. Strips positive for the presence of AIDS antibodies exhibit wide bands at the 41,000 molecular weight location.

The components of the Western Blot test kit include:

- 1) Nitrocellulose (or other polymeric matrix) sheets with the viral protein bands already resolved by gel-electrophoresis and transferred to the sheets as noted above and appropriately blocked with protein solutions to prevent further nonspecific protein binding;
- 2) A positive control human serum;

- 3) A negative control human serum;
- 4) A secondary antibody to human immunoglobulin raised in an appropriate animal host and tagged with a radioactive label;

5

- 5) Diluent solution;

- 6) A wash solution (0.5% sodium deoxycholate, 0.1M NaCl, 0.5% Triton X-100, 0.1mM phenylmethylsulfonyl fluoride and 10mM sodium phosphate; pH 7.5); and

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- 7) Test tubes with caps (e.g., 15cc).

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To use the Western Blot test kit, each strip is put in a 15cc tube containing 2.5 ml of the diluent and treated with 25  $\mu$ l of the test serum and incubated at 4°C overnight. The solution is drained and the strip is washed in the same tube with three changes of the wash solution, agitating well during the wash. 2.5 ml of the diluent is added to each tube and incubated for 1 hour at room temperature. To the contents of the tube is added 20  $\mu$ l of the radioactive secondary antibody solution (approximately  $2.5 \times 10^6$  dpm of  $^{125}$ I). After an additional incubation of 30 minutes at room temperature, the liquid is drained, the strips are again washed as above, the individual strips are removed from the tubes, blotted dry on a paper towel and mounted in order on filter-paper, wrapped with thin plastic film such as that of the Saran Wrap brand, and exposed to X-ray film in the dark. The film is developed after 24-48 hours. The antibodies reactive with the viral antigens are detected by dark images on the X-ray film. Positive antibody reactions to a 40-45,000 molecular weight antigen and a 23-25,000 molecular weight antigen are diagnostic of HTLV-III antibodies.

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Purified p24 may be radioactively labeled with  $^{125}$ I. Iodine is attached primarily to tyrosine residues of the protein. Radiolabeling procedures and radioimmunoassay (RIA) procedures may be performed in accordance with the methods described by Kalyanaraman et al in J. of Virology, 38(3):906-915, June 1981.

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The principle of this test procedure is based on the formation of immune complexes between antigens (generally proteinaceous materials or carbohydrate molecules or other macromolecules) and specific antibodies to the relevant antigens (for instance rabbit Ig against HTLV-III). These complexes are further reacted with a second antibody reactive against the first antibody used (for instance goat Ig against rabbit Ig). This reaction results in the formation of a ternary complex consisting of the antigen, the primary antibody and the secondary antibody. In addition, the primary antibody, which is always used in excess of that needed for complexing all the antigen present in the test, also forms binary complexes with the secondary antibody. The mixture of the complexes appears as a visible precipitate and can be sedimented by centrifugation. The antigen used in the test is radioactively labeled, usually with the isotope  $^{125}\text{I}$ . Therefore, the immune precipitation can be easily monitored by estimating the radioactivity associated with the precipitate by appropriate counting (e.g., by gamma counting for  $^{125}\text{I}$ -labeled antigen).

The components of the competition radioimmunoassay test kit for HTLV-III p24 include:

- (1) Labeled p24 (e.g.,  $^{125}\text{I}$ );
- (2) A primary antibody reactive with HTLV-III p24 (either hyperimmune antibody raised in an animal by inoculation with an antigen preparation containing HTLV-III p24, or a patient serum known to contain precipitating antibody against HTLV-III p24);
- (3) A secondary antibody against the primary antibody (e.g., antibody against rabbit immunoglobulins if the primary antibody used is a rabbit immunoglobulin, or antibody against human immunoglobulin if the primary antibody is a human serum reactive against HTLV-III p24);
- (4) The test material to be evaluated for the presence of HTLV-III p24;
- (5) pH buffers, surfactants, etc.

The procedure utilizing this test kit involves incubation of multiple dilutions of the non-radioactive test material with the primary antibody in separate test tubes for 1 hour prior to adding a constant amount of the radioactive antigen. The reaction mixture is then incubated initially at 37°C and subsequently at 4°C overnight. The secondary antibody is then added and the reaction mixture further incubated for 1 hour at 37°C followed by 2 hours at 4°C. The incubation mixture was then centrifuged to sediment the precipitate, the supernatant fluid aspirated out and the radioactivity in the precipitate determined using a gamma counter.

The radioactivity in the precipitate, in the reaction mixture not containing a competing antigen, is taken as the maximum precipitation of the labeled antigen. When preincubation of the primary antibody with an unlabeled antigen causes a reduction in the radioactivity associated with the precipitate, the conclusion is that the unknown test sample contains antigenic activity of HTLV-III p 24.

#### Detailed Description of the Figures

Figure 1 shows the identification of HTLV-III antigens by sera patients in accordance with the procedure of Example 2.

Figure 2 is a Western Blot analysis of antigenic cross-reactivities among HTLV-I, HTLV-II, and HTLV-III. Extracts of HTLV-I, HTLV-II, and HTLV-III were fractionated by SDS-polyacrylamide gel electrophoresis and the protein bands electroblotted to a nitrocellulose sheet. After blocking the remaining protein binding sites by incubation with 5% bovine serum albumin, the sheet was cut into segments, each containing lanes of HTLV-I, HTLV-II, and HTLV-III proteins. Segment A was incubated with a rabbit antiserum prepared against HTLV-I extract, segment B with a rabbit serum against HTLV-II extract, segment C with rabbit serum against HTLV-III extract and segment D with normal rabbit serum. After

washing, the segments were reacted with  $^{125}\text{I}$ -labeled goat IgG against rabbit immunoglobulin. The sheets were thoroughly washed, blotted dry and exposed to X-ray film. Lanes 1, 2, and 3 in each segment represent HTLV-I, HTLV-II, and HTLV-III. Numbers on the left represent molecular weights in thousands of standard marker proteins co-electrophoresed.

Figure 3 shows electrophoretic profiles of  $^{125}\text{I}$ -labeled p24 from HTLV-I, HTLV-II, and HTLV-III.  $^{125}\text{I}$ -labeled p24 proteins of HTLV-I (C), HTLV-II (B), and HTLV-III (A) were analyzed by electrophoresis through cylindrical 12% polyacrylamide gels in the presence of SDS. The gels were divided into 1 mm slices and counted in a gamma counter. The position of an  $^{125}\text{I}$ -labeled sample of chymotrypsinogen (mwt=25,500) in a parallel gel is indicated by the vertical dashed line.

Figure 4 shows the immunoprecipitation of  $^{125}\text{I}$ -labeled p24 of HTLV-III by sera of AIDS patients. Immunoprecipitations were performed by the double antibody method of Kalyanaraman et al, J. Virol., 38:906-915 (1981). Serial dilutions of sera were incubated with the labeled p24 of HTLV-III (approximately 8000 cpm) for 3 hours at  $37^\circ\text{C}$  followed by overnight at  $4^\circ\text{C}$  in a 200  $\mu\text{l}$  reaction mixture containing 20 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 0.3% Triton X-100, 2 mg/ml of bovine serum albumin, 1 mM EDTA and 0.1 mM PMSF. A 30-fold excess of goat anti-human IgG was then added and the incubation continued for 1 hour at  $37^\circ\text{C}$  and 2 hours at  $4^\circ\text{C}$ . The incubation mixture was diluted to 1 ml with the buffer and centrifuged at 4,000 rpm for 15 minutes in a Beckman TJ-6 centrifuge. The radioactivity bound in pellet was determined using a gamma counter.

Figure 5 shows the homologous competition radioimmunoassay for HTLV-III p24. The assay used a 1:1500 dilution of a hyperimmune rabbit serum prepared against disrupted HTLV-III. In the absence of competing unlabeled antigens, this serum dilution precipitated 20%



of the  $^{125}\text{I}$ -labeled p24. The rabbit serum was preincubated for 1 hour at  $37^\circ\text{C}$  with the unlabeled antigens listed, prior to adding the labeled antigen (approximately 8000 cpm). Further details of the assay are the same as described in the Figure 4 legend, except that the second antibody used was goat antirabbit immunoglobulin.

A. Competition with retroviral extracts:

●—●, HTLV-III; ●—●, HTLV-I; ○—○, HTLV-II; ▲—▲, FeLV; △—△, R-MuLV; ■—■, SSV; □—□, BaEV (M7); ▽—▽, SMRV; ▼—▼, MPMV; \*—\*, BLV; and x—x, EIAV p26.

B. Competition with cellular extracts:

●—●, H4 cells infected with HTLV-III; ○—○, uninfected H4 cells; □—□, uninfected H9 cells; ▲—▲, HUT 102 cells producing HTLV-I; and △—△, C3-44 cells producing HTLV-II.

Example 1

Antibodies to HTLV-III in Sera of Patients with AIDS and pre-AIDS Lymphadenopathy Syndrome

Wells of 96-well plates were coated overnight with a lysate of density-banded HTLV-III at 0.5  $\mu\text{g}$  protein per well in 100  $\mu\text{l}$  50 mM sodium bicarbonate buffer, pH 9.6. The wells were washed with water and incubated for 20 min. with 100  $\mu\text{l}$  of 5% bovine serum albumin in phosphate buffered saline (PBS). After washing, 100  $\mu\text{l}$  of 20% normal goat serum in PBS were added to each well, followed by 5 or 10  $\mu\text{l}$  of the test sera (blood taken from a human patient), and allowed to react for 2 hours at room temperature. The wells were washed three times with 0.5% Tween-20 in PBS in order to remove unbound antibodies, and were incubated for 1 hour at room temperature with peroxidase labeled goat anti-human IgG at a dilution of 1:2000 in 1% normal goat serum in PBS. Goat anti-human IgG is a second antibody that binds with the antibody-antigen complex formed in positive wells. The wells were successively washed 4 times with 0.05% Tween 20 in PBS and 4 times with PBS to remove unbound goat antibody,

and reacted with 100  $\mu$ l of the substrate mixture containing 0.05% orthophenylene diamine and 0.005% hydrogen peroxide in phosphate-citrate buffer, pH 5.0. This substrate mixture detects the peroxidase label and forms a colored product. The reactions were stopped by the addition of 50  $\mu$ l of 4N  $H_2SO_4$  and the color yield measured using an ELISA reader which quantifies the color reading. Assays were performed in duplicate; absorbance readings greater than three times the average of 4 normal negative control readings were taken as positive. The results are shown in Table 1.

Table 1

<u>Subjects</u>	<u>No. Positive/ No. Tested</u>	<u>Percent Positive</u>
Patients with AIDS	43/49	87.8
Pre-AIDS	11/14	78.6
Intravenous Drug abusers	3/5	60
Homosexual men	6/17	
Sexual contact with AIDS patient	1/1	
Persistent fatigue	1/1	
Other	4/15	26.6
Other controls	1/186	0.5
Normal subjects	1/164	0.6
Patients with Hepatitis B virus infection	0/3	0
Patients with Rheumatoid arthritis	0/1	0
Patients with Systemic lupus erythematosus	0/6	0
Patients with acute mononucleosis	0/4	0
Patients with lymphatic leukemias	0/8	0

Example 2

Western Blot analysis of the test area was conducted as follows. HTLV-III was lysed and fractionated by electrophoresis on a 12% polyacrylamide slab gel in the presence of sodium dodecylsulfate (SDS). The

protein bands on the gel were electrophoretically transferred to a nitrocellulose sheet, according to the procedure of Towbin et al., Proc. Natl. Acad. Sci. USA, 76:4350 (1979). Strip solid phase radioimmunoassays were then performed. The sheet was incubated at 37° for 2 hours with 5% bovine serum albumin in 10 mM Tris-HCl, pH 7.5 containing 0.9% NaCl and cut into 0.5 cm strips. Each strip was incubated for 2 hours at 37° and 2 hours at room temperature in a screw cap tube containing 2.5 ml of buffer 1 (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2 M NaCl, 0.3% Triton X-100 and 2 mg/ml bovine serum albumin and 0.2 mg/ml of human antibody fractions, Fab). Test sera (25 µl), taken from human patients with AIDS or exhibiting pre-AIDS symptoms, were then added to individual tubes containing the strips and incubation continued for 1 hour at room temperature and overnight in the cold. The strips were washed three times with solution containing 0.5% sodium deoxycholate, 0.1 M NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 10 mM sodium phosphate, pH 7.5. The strips were incubated for 1 hour at room temperature with 2.4 ml of buffer 1 and 0.1 ml of normal goat serum. Affinity purified and <sup>125</sup>I-labeled goat anti-human immunoglobulin (µ chain and Fc fragment) (1.25 x 10<sup>6</sup> cpm) were added to the reaction mixture and the incubation continued for 30 minutes at room temperature. The strips were washed as described, dried, mounted and exposed to X-ray film. Figure 1 indicates graphically the results of these experiments. Strip 1 is test sera from an adult with T-cell leukemia; Strip 2 is a normal donor; Strip 3 is a mother of a child with AIDS; Strips 4 and 6-10 are AIDS patients; and Strip 5 is a patient with pre-AIDS.

### Example 3

#### Fixed Cell and Live Cell Indirect Immunofluorescence Assay for Antibodies to HTLV-III

Indicator cell: HTLV-III infected negative cells; negative control: uninfected T-cells.

Infected cells were washed with phosphate buffered saline (PBS) and resuspended in PBS at  $10^6$  cells/ml. Approximately 50 ml of cell suspension were spotted on a slide, air dried, and fixed in acetone for 10 minutes at room temperature. Slides were stored at  $-20^{\circ}\text{C}$  until ready for use. 20 ml of the test human serum diluted 1:10 in PBS were added to the fixed cells and incubated for 1 hour at  $37^{\circ}$ . Slides were washed and reacted for 30 minutes at room temperature with a dilute solution of fluorescein-conjugated goat anti-human IgG. Slides were again washed and examined under a microscope for fluorescence. A negative control used uninfected parental cells. The above describes a fixed cell system in which the antibody antigen reaction is sought for both inside and outside the cell.

For live cell immunofluorescence assay all the above reactions were carried out in a tube instead of on a slide, but without chemical fixation of the cells. After reaction with the fluorescein conjugated anti-human antibody, the cells were added to the slide and examined under a microscope for antibody-antigen reaction on the surface of the cell.

The results of each of these assays show a strong fluorescence reaction specifically with sera of AIDS and pre-AIDS patients.

#### Example 4

Sera of patients with clinically documented AIDS, Kaposi's sarcoma, sexual contacts with AIDS patients, intravenous drug abusers, homosexual men and heterosexual donors were tested for their reactivity to HTLV-III. The system employed was ELISA. Lysates of sucrose density banded HTLV-III were coated on 96-well microtiter plate wells. Test sera were diluted with a dilute solution of normal goat serum, added to the wells, and allowed to react for 2 hours or overnight at room temperature. The antibodies in the human sera were detected by the reaction of the primary immune complex

with peroxidase labeled goat anti-human immunoglobulins followed by the development of a colored peroxidase reaction product. The results obtained are presented in Table 1. Of 49 clinically diagnosed AIDS patients, 43 (88%) showed serum reactivity in this assay. Fourteen homosexual men with pre-AIDS were also tested for anti-bodies to HTLV-III. Of these, 11 (79%) were positive. Among 17 homosexual men with no clinical symptoms of AIDS, 7 scored positive. Of these, at least one was known to be a long-time sexual partner of a diagnosed case of AIDS. One had persistent fatigue and was exhibiting early signs of AIDS. One of the three intravenous drug abusers that were positive for serum anti-bodies to HTLV-III was also homosexual.

In contrast, only 1 of 186 controls tested reacted positive in this test. They included 3 with hepatitis B virus infection, 1 with rheumatoid arthritis, 6 with systemic lupus erythematosus, 4 with acute mononucleosis and 8 with various forms of lymphatic leukemias and lymphomas, some of whom were positive for HTLV-I. The remaining test subjects were normal donors of unknown sexual preference, including laboratory workers ranging in age from 22 to 50.

#### Example 5

To investigate the specificity of the reactions, lysates of the HTLV-III-infected cell clones were analyzed in comparison with lysates of the same cell clones before viral infection. No reactive antigen was found in the uninfected clones, with the exception of an 80,000 molecular weight band in H17 which bound antibodies from all human sera tested, but not from rabbit or goat serum. Antigens newly expressed after viral infection and recognized by the human serum used for this analysis include p65, p55, p41, p39, p32 and p24. In addition, a large protein of approximately 130,000 molecular weight and one of 48,000 molecular weight were detected. With normal human serum, none of the antigens

was detected. These results show that the antigens detected are either virus-coded proteins or cellular antigens specifically induced by viral infection.

Example 6

Sucrose density banded HTLV-III from culture supernatants of H9/HTLV-III cells was analyzed by the Western blot technique for determination of its antigenic similarities with HTLV-I and HTLV-II. Lysates of HTLV-I, HTLV-II, and HTLV-III were fractionated by SDS-polyacrylamide gel electrophoresis and electroblotted to a nitrocellulose sheet. The protein bands were then reacted with rabbit hyperimmune sera to HTLV-I, HTLV-II, or HTLV-III. The results are shown in Figure 2. The strongest immune reactivity for p24 was observed with the homologous antisera (Lanes A-1, B-2, and C-3). Moderately strong cross-reactivities were seen between HTLV-II p24 and anti-HTLV-I (Lane A2), and HTLV-III p24 and anti-HTLV-II (Lane B3). Weak reactivities were also seen between HTLV-III p24 and anti-HTLV-I (Lane A3), and HTLV-I p24 and an anti-HTLV-III (Lane C1). However, in spite of the moderate reactivity between HTLV-III p24 and anti-HTLV-II (Lane B3), no reciprocal reactivity between HTLV-II p24 and anti-HTLV-III (Lane C2) was found. No reactivity was observed with normal rabbit sera (Panel D).

The major HTLV-III core protein, p24, was purified from the lysate of a sucrose density banded virus preparation. The virus was solubilized with 0.5% Triton X-100, 0.8M NaCl, 20% glycerol and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in 50 mM Tris-HCl, pH 7.9, and the extract was freed of nucleic acids by a batch adsorption and elution from DEAE-cellulose with 0.3M NaCl. The eluate was dialyzed against 50 mM BES buffer, pH 6.5 containing 1 mM EDTA, 0.1 mM PMSF and chromatographed on a phosphocellulose column equilibrated with the same buffer. The proteins were eluted using a linear 0 to 0.6 M NaCl gradient. HTLV p24 eluted between 0.25 and 0.3 M NaCl. Figure 3 shows the SDS-polyacrylamide gel profile

of a sample after labeling with  $^{125}\text{I}$ . In electrophoretic mobility, HTLV-III p24 was very similar to HTLV-I p24 and HTLV-II p24, and significantly faster than  $^{125}\text{I}$ -labeled chymotrypsinogen run in a parallel gel as a molecular weight marker ( $\text{mwT}=25,500$ ) (Figure 2, vertical dashed line).

The specificity of the purified p24 was analyzed by immunoprecipitation with patient sera and by competition radioimmunoassays using hyperimmune rabbit sera.

Figure 4 shows the results of immunoprecipitation of  $^{125}\text{I}$ -labeled p24 of HTLV-III with sera of patients with AIDS and AIDS related conditions. A wide range of antibody titers was evident from these experiments and this finding agreed with results from Western Blot analysis that indicated that immune reactivities with p24 tended to vary widely and that sera of some AIDS patients did not show significant antibody reactivity with p24.

A homologous competition radioimmunoassay was set up using  $^{125}\text{I}$ -labeled HTLV-III p24 and a rabbit antibody raised against disrupted HTLV-III. Figure 5A shows a total competition of the precipitation of the labeled p24 by unlabeled HTLV-III extract. Extracts of HTLV-I and HTLV-II showed only a minimal competition at protein concentrations about 100-fold higher than HTLV-III. On the other hand, a large number of other mammalian retroviruses (Feline leukemia virus (FeLV), Rauscher murine leukemia virus (R-MuLV), simian sarcoma virus (SSV), baboon endogenous virus (BaEV), squirrel monkey retrovirus (SMRV), Mason-Pfizer monkey virus (MPMV), and bovine leukemia virus (BLV)) did not compete in this immunoprecipitation. Similarly, two cell clones infected with HTLV-III (H4/HTLV-III and H9/HTLV-III) blocked the immunoprecipitation of HTLV-III p24, but the corresponding uninfected cells were totally without effects (Fig. 5B). Cells producing HTLV-I and HTLV-II exhibited marg.-

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nal effects in this competition at relatively higher protein concentrations. Therefore, the antigens involved in the above reactions were specific to HTLV-III and cells producing HTLV-III. There were detectable, although low level, cross-reactivities between HTLV-III p24 and p24 of HTLV-I and HTLV-II. These cross-reactivities became more easily evident in a less stringent system such as Western Blots (Figure 2). Purified p26 of equine infectious anemia virus (EIAV) here showed little cross-reactivity in the homologous radioimmunoassay for HTLV-III p24 (Figure 5A). The effect of HTLV-III was checked in homologous competition radioimmunoassays for HTLV-I p24 and HTLV-II p24. While these systems showed considerable cross-reactivity between HTLV-I and HTLV-II, competition by HTLV-III was only minimal in either assay.

The results showed that HTLV-III is a unique retrovirus with a major core protein, p24, unrelated to most other retroviruses. The p24 of HTLV-III, however, shares a low but detectable level of antigenic cross-reactivity with HTLV-I and HTLV-II and not with other retroviruses. These cross-reactivities with HTLV-I and HTLV-II were seen more clearly in the Western Blot analysis than in conventional competition radioimmunoassays. It was analogous to the detection of cross-reactivities between HTLV-I p24 and BLV p24 by the Western Blot, although no such cross-reactivities could be demonstrated between the two viruses using competition radioimmunoassays. It was clear that HTLV-I and HTLV-II were more closely related to each other than HTLV-III was to either HTLV-I or HTLV-II. These findings were in agreement with the detection of a limited nucleic acid sequence homology between HTLV-III and both HTLV-I and HTLV-II, especially in the gag-pol region of the genomes.

#### Example 7

Therapeutic AIDS-specific test kits were constructed for detecting antibodies using several different techniques for detection. One test kit for antibodies



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detection comprised a compartmented enclosure containing a plurality of wells, plates which were coated prior to use with p24 of HTLV-III and ELISA materials for enzyme detection consisting of normal goat serum and peroxidase-labeled goat antihuman IgG and a color change indicator consisting of orthophenylene diamine and hydrogen peroxide in phosphate citrate buffer.

5 A second test kit for detecting antibodies using the Dot Blot technique comprised a container, a cover, and therein containing a nitrocellulose sheet with HTLV p24 attached by "dot-blotting," and additionally surfactants as well as pH modifiers and bovine serum albumin and human Fab. This Dot Blot analysis container also contained a supply of dilute normal goat serum and  $^{125}\text{I}$  labeled goat antihuman immunoglobulin.

10 Finally, a different AIDS-specific test kit for detecting HTLV-III using competition radioimmunoassay comprised a compartmented container,  $^{125}\text{I}$ -labeled p24 of HTLV-III, rabbit (or goat) anti-HTLV-p24, solutions of surfactants, pH buffers, bovine serum albumin and goat antiserum to rabbit immunoglobulin (or rabbit antiserum to goat immunoglobulin).

WHAT IS CLAIMED IS:

1. A method for detection of HTLV-III virus, close derivatives of HTLV-III virus, antibodies to HTLV-III virus or antigens of HTLV-III virus in a sample, characterized in that said sample is subjected to a competition immunoassay discrimination procedure.

2. A method according to claim 1, characterized in that HTLV-III virus is used to detect antibodies to HTLV-III virus in said sample.

3. A method according to claim 1, characterized in that an antigen of HTLV-III virus is used to detect antibodies to HTLV-III virus in said sample.

4. A method according to claim 2 or 3, characterized in that said discrimination procedure is carried out in the presence of HT neoplastic aneuploid T-cells.

5. A method according to claim 3, characterized in that said antigen is the envelope antigen p 41 of HTLV-III virus.

6. A method according to claim 3, characterized in that said antigen is the core antigen p 24 of HTLV-III virus.

7. A method according to claim 1, characterized in that labelled HTLV-III virus, labelled close derivatives of HTLV-III virus or labelled antigens of HTLV-III virus are used in said discrimination procedure.

8. A method according to claim 1, characterized in that labelled antibodies to HTLV-III virus are used in said discrimination procedures.

9. A method according to claim 7 or 8, characterized in that HTLV-III virus, close derivatives of HTLV-III virus or antigens of HTLV-III virus in said sample are detected by said discrimination procedure.

10. A method according to claim 6, characterized in that said discrimination procedure is selected from ELISA and immunoprecipitation assay methods.

11. A method according to claim 2, 3 or 5, characterized in that said discrimination procedure is

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selected from ELISA, Western Blot and immunofluorescence assay methods.

5 12. A method according to claim 11, characterized in that said discrimination procedure is an ELISA assay method.

10 13. A method according to claim 1, characterized in that an antibody to HTLV-III virus is reacted with an antigen of HTLV-III virus to form an immune complex, and said immune complex is reacted with a labelled secondary antibody to produce a readily quantifiable product.

14. A method according to claim 13, characterized in that said secondary antibody is tagged with an enzyme which can produce a colored product.

15 15. A method according to claim 13, characterized in that said secondary antibody is tagged with radioactive  $^{125}\text{I}$ .

20 16. A method according to claim 15, characterized in that said antigen is the core antigen p 24 of HTLV-III virus.

25 17. A method according to claim 3, characterized in that said discrimination procedure is of the Western Blot type wherein protein bands of HTLV-III virus are formed by electrophoresis of HTLV-III virus on a polyacrylamide gel in the presence of sodium dodecylsulfate, the protein bands in the gel are transferred to a nitrocellulose sheet, the sheet is saturated with an unrelated protein, the sheet is separated into strips representing the profile of the antigens to HTLV-III virus, and the antigens are used for detecting antibodies in said sample.

18. A method according to claim 17, characterized in that said unrelated protein is bovine albumin.

35 19. A method according to claim 3, characterized in that said antigen is obtained by concentrating HTLV-III virus by ultracentrifugation from virus culture supernatants, removing lipids by centrifugation through

20% (W/W) sucrose in TNE buffer to form a sucrose gradient, dividing the sucrose gradient into fractions, and isolating antigen bands by assaying aliquots of each fraction for HTLV-III virus specific reverse transcriptase activity.

20. A method according to claim 7, characterized in that labelled core antigen p24 of HTLV-III virus is used in said discrimination procedure, said p24 antigen being purified and labelled by

- a) centrifuging to obtain a virus culture supernatant and remove cells;
- b) concentrating the virus in sucrose;
- c) adding a non-ionic detergent with sodium chloride to free the nucleic acids inside the virus;
- d) chromatographing using DEAE cellulose and then chromatographing using phosphocellulose to recover p24 core antigen; and
- e) labeling the protein using an iodine isotope such as  $^{125}\text{I}$ .

21. A method according to claim 7 or 8, characterized in that said sample comprises tissue culture material or primary cells of body fluids.

22. A method according to claim 7 or 8, characterized in that said sample comprises sera or a synthetic blood product.

23. An AIDS specific test kit comprising a compartmented enclosure, characterized in that at least one compartment of said enclosure contains at least one of HTLV-III virus, close derivatives of HTLV-III virus, antibodies to HTLV-III virus and antigens of HTLV-III virus.

24. A test kit according to claim 23, characterized in that said at least one compartment contains antibodies to HTLV-III virus.

25. A test kit according to claim 23, characterized in that said at least one compartment contains HTLV-III virus.

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26. A test kit according to claim 23, characterized in that said at least one compartment contains antigens of HTLV-III virus.

5 27. A test kit according to claim 26, characterized in that said antigens are core antigens p24 of HTLV-III virus.

28. A test kit according to claim 26, characterized in that said antigens are envelope antigens p41 of HTLV-III virus.

10 29. A test kit according to claim 23, characterized in that said at least one compartment contains HTLV-III virus lysate.

30. A test kit according to claim 29, characterized in that it includes

15 a) microtiter plates coated with HTLV-III virus lysate and treated with protein solutions to prevent further adsorption of proteins to the plates by means other than antigen-antibody interaction;

20 b) control positive human serum of known reactivity and antibody titer to antigens of HTLV-III virus;

c) negative control serum with no antibody titer to antigens of HTLV-III virus;

25 d) enzyme-labelled secondary antibody reactive against human immunoglobulins, and a diluent solution for the secondary antibody consisting of normal serum of the animal species in which the secondary antibody is raised;

e) a substrate mixture of orthophenylene-diamine and  $H_2O_2$ ;

30 f) a solution to stop the enzyme reaction;

g) washing buffer; and

h) phosphate buffered saline.

35 31. A method according to claim 27, characterized in that said enclosure contains a plurality of wells, plates which are coated prior to use with the core antigen p24 of HTLV-III virus, and ELISA materials for enzyme detection consisting of normal goat serum and peroxidase-labeled goat antihuman IgG and a color change

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indicator consisting of orthophenylene diamine and hydrogen peroxide in phosphate citrate buffer.

5 32. A test kit according to claim 31, characterized in that core antigen p24 of HTLV-III is present in the form of a lysate.

10 33. A test kit according to claim 27, characterized in that said enclosure contains  $^{125}\text{I}$ -labeled p24 antigen of HTLV-III, rabbit or goat anti-HTLV-p24, solutions of surfactants, pH buffers, bovine serum albumin and goat antiserum to rabbit immunoglobulin or rabbit antiserum to goat immunoglobulin.

15 34. A test kit according to claim 25, characterized in that said enclosure contains a plurality of wells, plates which are coated prior to use with HTLV-III and ELISA materials for enzyme detection consisting of normal goat serum and peroxidase, labeled goat antihuman IgG and a color change indicator.

20 35. A test kit according to claim 34, characterized in that said color change indicator consists of orthophenylene diamine and hydrogen peroxide in phosphate citrate buffer.

25 36. A test kit according to claim 35, characterized in that said HTLV-III is present in the form of a lysate.

37. A test kit according to claim 25, characterized in that said enclosure further contains phosphate buffered saline and fluorescein-conjugated goat antiserum IgG.

30 38. A test kit according to claim 25, characterized in that said enclosure further contains a nitrocellulose sheet and a polyacrylamide slab gel in the presence of sodium dodecylsulfate, surfactants, pH modifiers, bovine serum albumin, human Fab, and a supply of dilute normal goat serum and  $^{125}\text{I}$  labeled goat antihuman immunoglobulin.

35 39. A test kit according to claim 28, characterized in that said enclosure contains

a) nitrocellulose sheets with HTLV-III protein bands already resolved by gel-electrophoresis and appropriately blocked with protein solutions to prevent further nonspecific protein binding;

b) a positive control human serum;

c) a negative control human serum;

d) a secondary antibody to human immunoglobulin raised in an appropriate animal host and tagged with a radioactive label;

e) diluent solution;

f) wash solution;

g) test tubes with caps.

40. A test kit according to claim 39, characterized in that the wash solution is 0.5% sodium deoxycholate, 0.1M NaCl, 0.5% Triton X-100, 0.1mM phenylmethylsulfonyl fluoride and 10mM sodium phosphate; pH 7.5.

41. A test kit according to claim 27, characterized in that said enclosure further contains primary antibodies reactive with said core antigens p24, secondary antibodies against the primary antibodies, pH buffers and surfactants.

42. A test kit according to claim 41, characterized in that said enclosure contains a reaction vessel for addition of test material to be evaluated for core antigens p24.

43. A test kit according to claim 41, characterized in that said primary antibodies are hyperimmune antibodies raised in an animal by inoculation with an antigen preparation containing HTLV-III virus core antigen p24.

44. A test kit according to claim 43, characterized in that said primary antibodies are rabbit immunoglobulins and said secondary antibodies are antibodies against rabbit immunoglobulin.

45. A test kit according to claim 41, characterized in that said primary antibodies are contained in

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a patient serum known to contain precipitating antibodies against HTLV-III core antigen p24.

46. A test kit according claim 45, characterized in that said secondary antibodies are antibodies  
5 against human immunoglobulin.



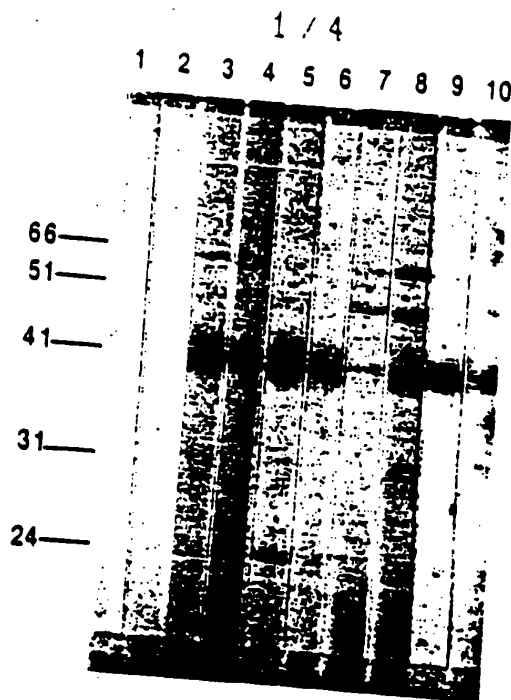
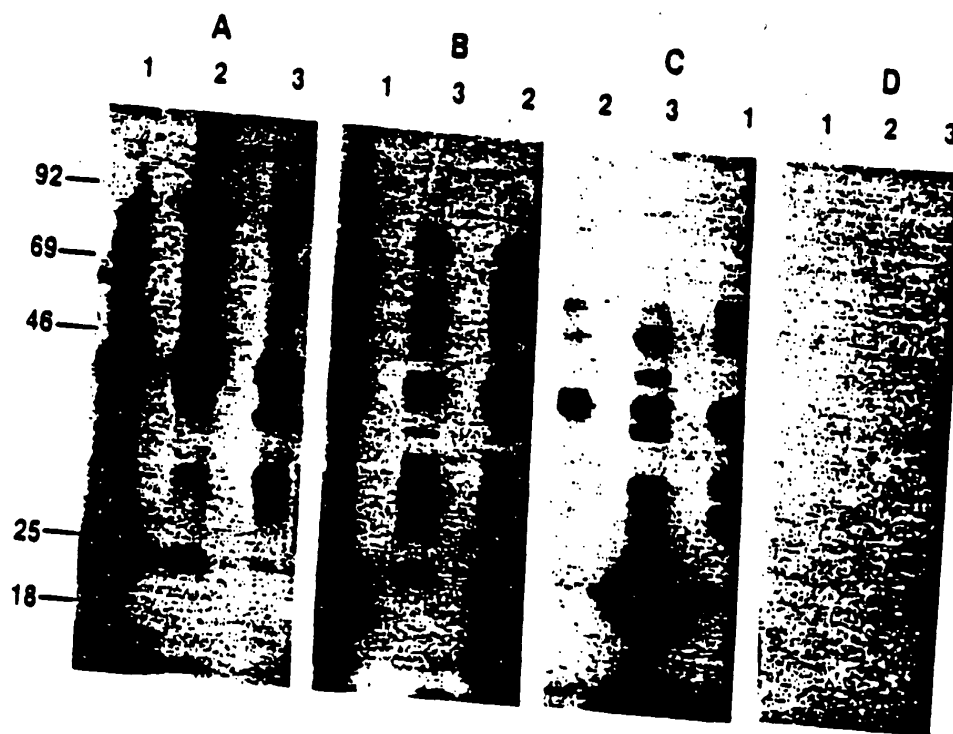


FIG. 1



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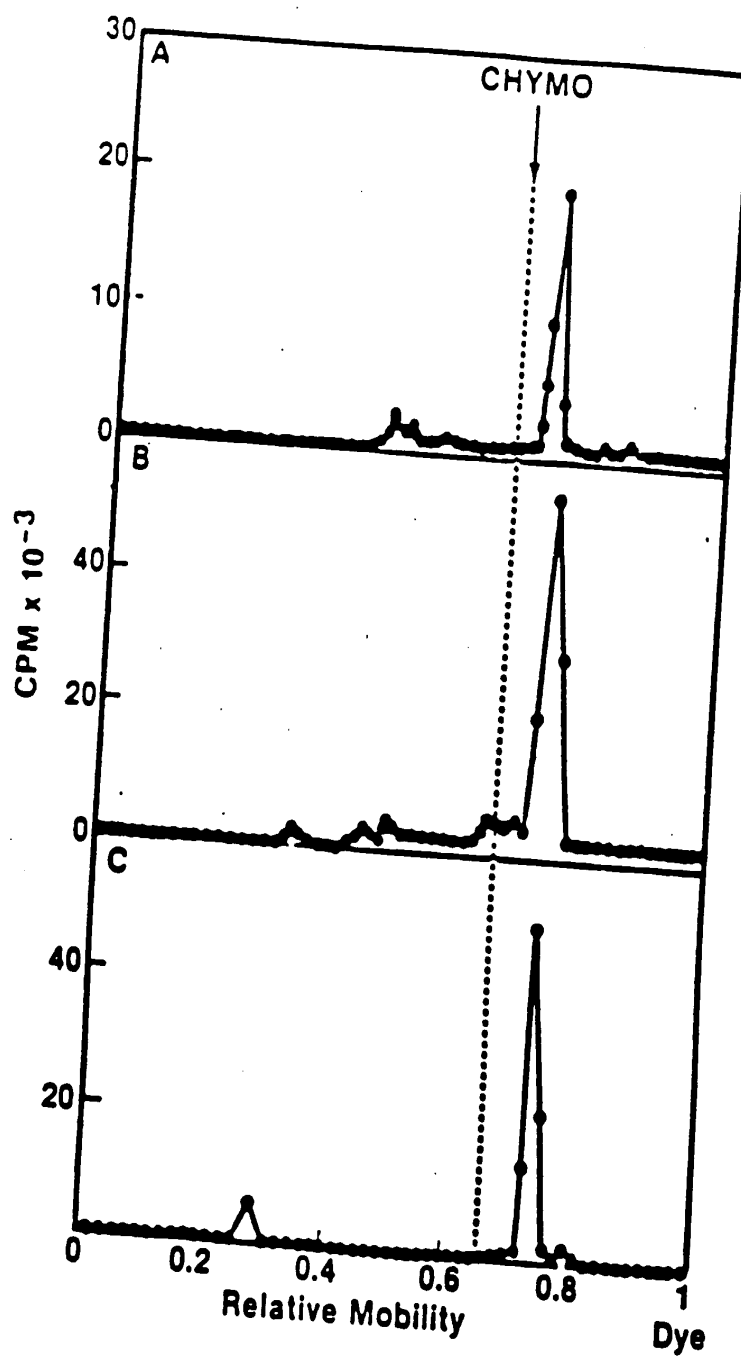


FIG.3

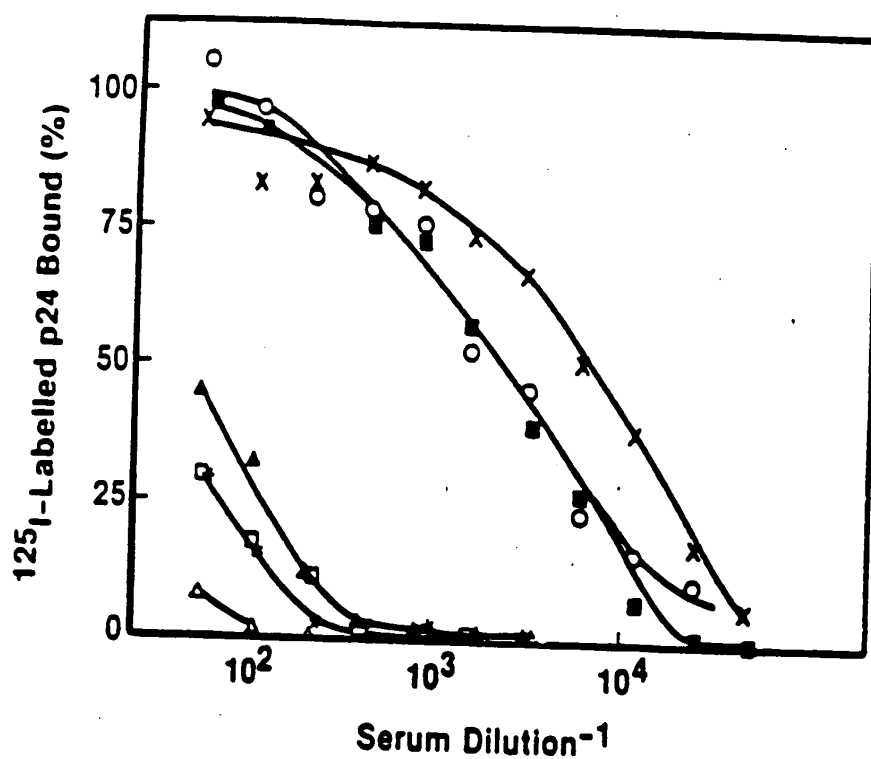


FIG. 4

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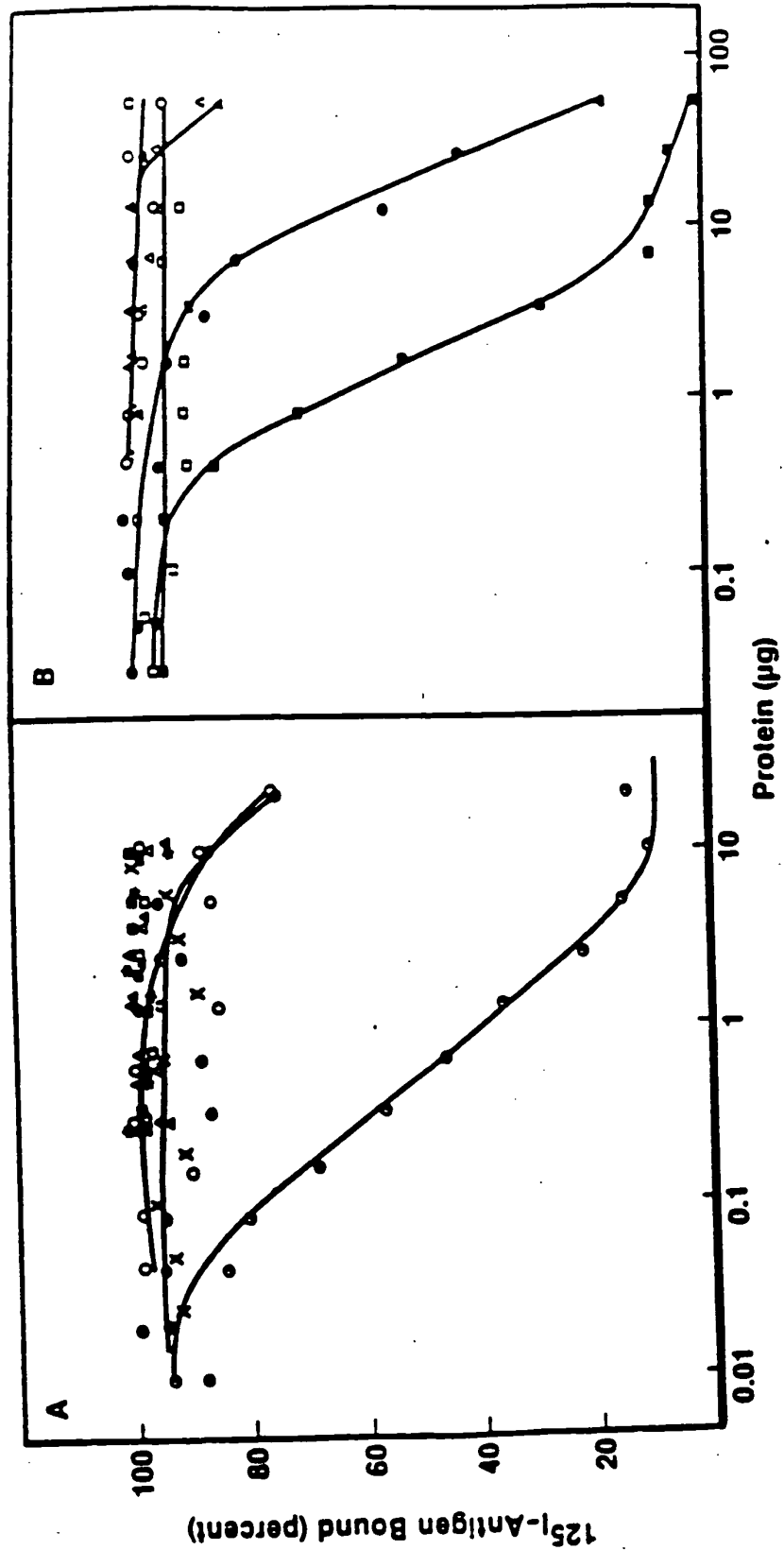


FIG. 5